AFRL-OSR-VA-TR-2013-0553

REPROGRAMMING MICROBES FOR THE REMOTE DETECTION OF ENVIRONMENTAL THREATS

JUSTIN GALLIVAN

EMORY UNIVERSITY

10/15/2013
Final Report

AIR FORCE RESEARCH LABORATORY
AF OFFICE OF SCIENTIFIC RESEARCH (AFOSR)/RSL
ARLINGTON, VIRGINIA 22203
AIR FORCE MATERIEL COMMAND

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ORGANIZATION.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
14/10/2013	Final	16/07/2010-15/7/2013

4. TITLE AND SUBTITLE

Reprogramming Microbes for the Remote Detection of Environmental Threats

5a. CONTRACT NUMBER

5b. GRANT NUMBER

FA9550-10-1-0359

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Justin Gallivan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Emory University Office of Grants and Contracts Accounting
1599 Clifton Road, 3rd Floor
Atlanta, GA 30322

8. PERFORMING ORGANIZATION REPORT NUMBER

0000004509

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

USAF, AFRL
Air Force Office of Scientific Research
875 N. Randolph Street, Rm. 312
Arlington, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)

USAF/AFRL/AFOSR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

FA9550-10-1-0359

12. DISTRIBUTION/AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Riboswitches are sequences of RNA that control gene expression via RNA-ligand interactions, without the need for accessory proteins. Riboswitches consist of an aptamer that recognizes the ligand and an expression platform that couples ligand binding to a change in gene expression. Using in vitro selection, it is possible to screen large (~10^13 member) libraries of RNA sequences to discover new aptamers. However, limitations in bacterial transformation efficiency make screening such large libraries for riboswitch function in intact cells impractical.

We established that an E. coli S30 extract that couples transcription/translation is sufficient to recapitulate the function of riboswitches expressed in intact cells. We established that a family of synthetic riboswitches acts through a kinetic trapping mechanism, rather than an equilibrium mechanism. Finally, we established CIS-Select as an in vitro system to perform selections for novel synthetic riboswitches. We anticipate that with optimization, CIS-Select will enable direct selection for riboswitch activity from very large RNA libraries, while bypassing the limitations of bacterial transformation efficiency.

15. SUBJECT TERMS

riboswitches, gene expression, synthetic biology

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(Include area code)*

Final Technical Report for Grant FA9550-10-1-0359
Reprogramming Microbes for the Remote Detection of Environmental Threats
Justin Gallivan, Emory University

Program Director: Dr. Hugh DeLong, AFOSR

Abstract

Riboswitches are sequences of RNA that control gene expression via RNA-ligand interactions, without the need for accessory proteins. Riboswitches consist of an aptamer that recognizes the ligand and an expression platform that couples ligand binding to a change in gene expression. Using in vitro selection, it is possible to screen large (~10^{13} member) libraries of RNA sequences to discover new aptamers. However, limitations in bacterial transformation efficiency make screening such large libraries for riboswitch function in intact cells impractical.

To address this, we established that an *E. coli* S30 extract that couples transcription/translation is sufficient to recapitulate the function of riboswitches expressed in intact cells. Through mechanistic studies, we established that a family of synthetic riboswitches acts through a kinetic trapping mechanism, rather than an equilibrium mechanism. Finally, we established CIS-Select as an in vitro system to perform selections for novel synthetic riboswitches. We anticipate that with optimization, CIS-Select will enable direct selection for riboswitch activity from very large RNA libraries, while bypassing the limitations of bacterial transformation efficiency.

We welcome the opportunity to report our progress during the granting period.

The original goals of the proposal involved developing synthetic riboswitches that detect the presence of new small molecules (such as organophosphate nerve agents), and to use these riboswitches to program bacteria to perform complex tasks, such as forming patterns in the presence of the target. These were ambitious goals, and although we made some progress during the first 18 months of the 36 month granting period, we realized that developing new synthetic riboswitches would require laying some groundwork through fundamental studies. After our annual progress meeting with Dr. DeLong in 2011, we decided to focus our efforts on determining what characteristics make for a 'good' riboswitch, with the goal of making riboswitch selection easier.

Riboswitches must solve two chemical problems: binding the ligand, and coupling ligand-binding into a change in gene expression. To first order, ligand binding can be considered a thermodynamically-controlled (equilibrium) process, while the change in gene expression is a kinetically controlled process. Previous efforts toward riboswitch discovery have focused on solving the first problem by developing tight-binding aptamers through in vitro selection. The second problem, kinetic control, has typically been addressed in an *ad hoc* manner using screens and selections for function. We asked whether it would be possible to perform direct in vitro selections for riboswitch activity.

To do this, we needed to know whether riboswitches would function in an in vitro system in the same manner that they performed in cells. Previous studies from our lab revealed an equilibrium mechanism for riboswitch function, so we expected that RNA templates added to an *E. coli* S30 translation extract would perform well—activating protein expression when the ligand (in this case, theophylline) was added. Surprisingly, this did not occur (Figure 1).

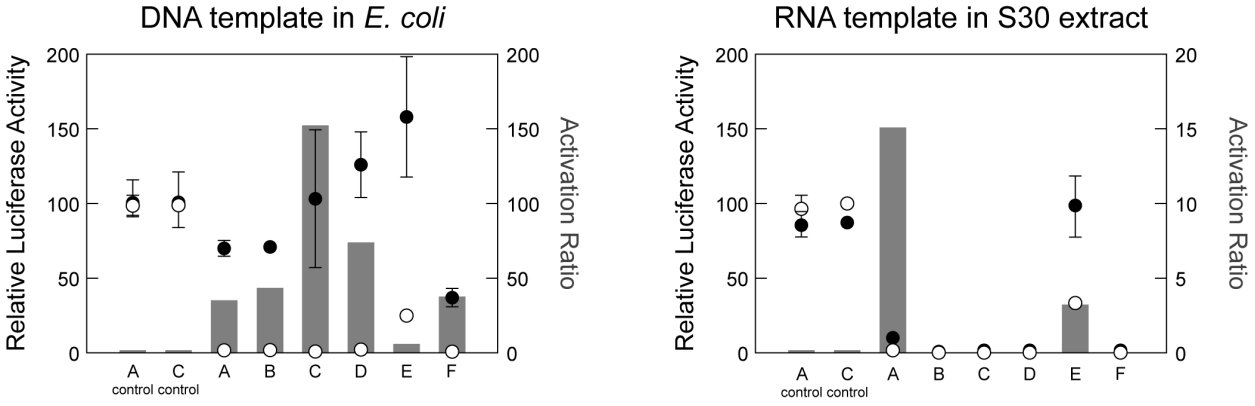

Figure 1. Comparison of a family of synthetic riboswitches expressed from a plasmid in intact *E. coli*, or from an mRNA template in *E. coli* S30 extract. *Left axis:* Black dots: 2 mM theophylline; white dots: no theophylline. *Right axis:* Gray bars: activation ratio determined by dividing expression with theophylline by expression without. The function of the riboswitches is compromised when they are introduced as RNA templates in extract.

Although the mRNA templates did not function well in S30 extract, we showed that DNA templates added to an S30 extract capable of performing coupled transcription and translation restored riboswitch performance (not shown). This suggested that there might be a co-transcriptional component to the mechanism. To test this, we decoupled the processes of transcription and translation as shown in Figure 2.

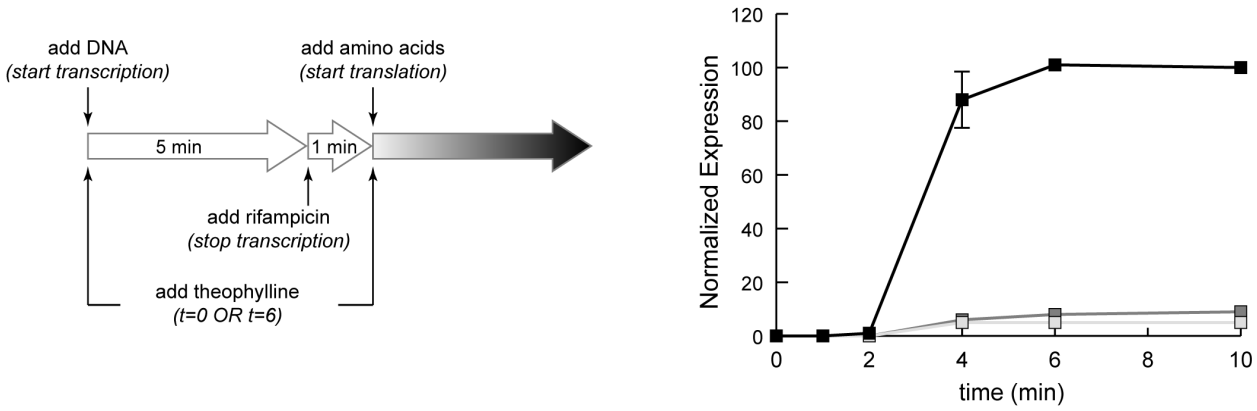

Figure 2. Decoupling transcription and translation. *Left:* Schematic of decoupling transcription and translation in S30 extract. Theophylline (2 mM) is added at t=0 min (present for transcription and translation) or t=6 min (present for translation only). The expression of the luciferase reporter is monitored as a function of time after the addition of the amino acids (the start of translation). *Right:* results from an experiment performed as shown in the left panel. No theophylline added (light gray squares), theophylline added only after transcription was terminated (dark gray squares), theophylline present during transcription and translation (black squares).

We discovered that, although this riboswitch regulates translation, the 'decision' to regulate occurs co-transcriptionally. Thus, the system is not at equilibrium, and the ligand must be present during the time that the RNA is being synthesized and is folding.

This is a key result, as it suggests that our previous strategies for riboswitch selection may have been misguided. For example, we (and others in the field) generally believed that to get a riboswitch that functioned well, it would be critical to have a tight-binding aptamer. However, tight-binding can result from a combination of two things: fast ligand association kinetics and/or slow dissociation kinetics. While diffusion imposes a speed limit to association, dissociation kinetics can be arbitrarily long (i.e., the aptamer binds the ligand and never lets it go). Because transcription is fast (these riboswitches are likely transcribed in under 5 seconds), and mRNA lifetime is relatively short (on the order of a few minutes), overly-long dissociation kinetics don't buy us much, and may hurt us.

This work (along with additional mechanistic details) has been submitted to *Nucleic Acids Research*. "A Family of Synthetic Riboswitches Adopts a Kinetic Trapping Mechanism", D.M. Mishler and J.P. Gallivan.

After we showed that riboswitches function in a coupled in vitro transcription/translation system, we could ask whether we could use such a system (often referred to as Tx/Tl) to perform direct selections for riboswitch activity. The ability to use Tx/Tl is critical, as it increases the library sizes that we can screen by >5 orders of magnitude. In past experiments, we were limited by the transformation efficiency of *E. coli*, which is ~10^8 transformants/experiment. However, in vitro selection for aptamers typically uses library sizes >10^{13} members.

To address these issues, we developed a new method for riboswitch selection called CIS-Select, which is shown schematically in Figure 3.

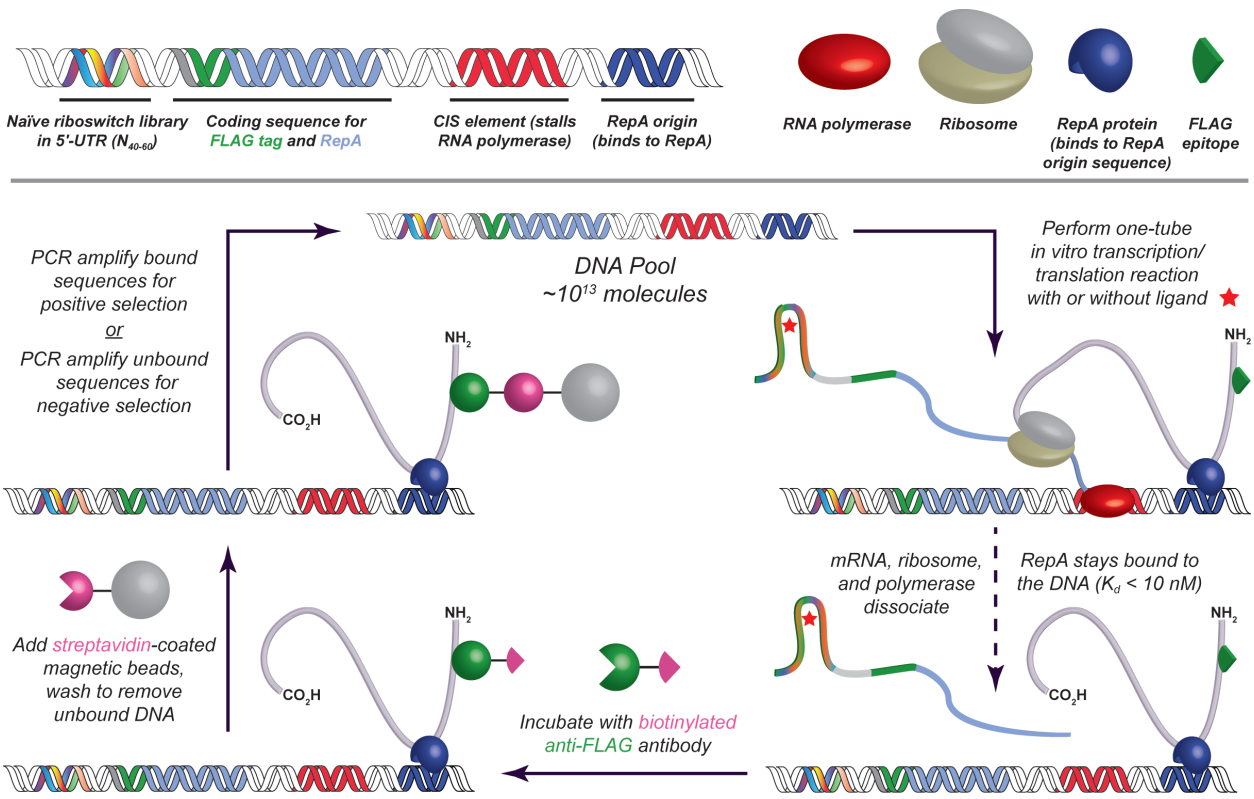

Figure 3. *Top:* Outline of components used in CIS-select. The initial template DNA library is prepared synthetically, using random nucleotides in the 5′-UTR. *Bottom:* CIS-select workflow for selecting ligand-activated riboswitches. DNA is added to an in vitro transcription/translation system with the ligand (★). If the sequence is translated efficiently, a FLAG-tagged RepA protein binds to the DNA that encoded it; the protein/DNA complex is captured with antibody, and amplified by PCR. Sequences that don't translate efficiently not selected. Performing a cycle *without* ligand and amplifying *unbound* DNA selects against constitutively activated sequences.

CIS-Select, which is based, in part, on a previously reported strategy for protein engineering known as CIS display[1], relies on two observations: An RNA sequence known as the CIS element pauses transcription; and a protein known as RepA binds to a DNA sequence known as the RepA origin. When a DNA that encodes the coding sequence of repA, the CIS element, and the RepA origin is expressed in Tx/Tl, the RepA protein is synthesized, and folds while the RNA polymerase is stalled at the CIS element. The RepA protein then binds to the RepA origin on the DNA, providing a physical linkage between a protein and the DNA that encoded it.

We modified the system so the protein component also encodes a FLAG epitope, so we can pull down sequences that are expressed. We also introduced DNA encoding a randomized riboswitch in 5'-untranslated region. The idea is if the switch is on, the FLAG epitope will be expressed and we will

[1] R. Odegrip, D. Coomber, B. Eldridge, R. Hederer, P. A. Kuhlman, C. Ullman, K. FitzGerald, D. McGregor, CIS display: In vitro selection of peptides from libraries of protein-DNA complexes. *Proc. Natl. Acad. Sci. USA* **101**, 2806 (2004).

capture the DNA of the library member that encoded it (Figure 3). If the switch is off, we can select for DNAs that do not get pulled down with a anti-FLAG antibody. By performing positive and negative selections in the presence of the ligand, we can theoretically screen large (10^{13} member) DNA libraries for riboswitch function.

CIS-Select has some other notable advantages over previous methods of obtaining riboswitches. For example, CIS-select selects for switching directly, rather than in a two-step process involving aptamer selection, followed by grafting on the riboswitch expression platform. Moreover, the process does not require coupling the ligand to a solid support—this reduces the demand for chemical synthesis *and* allows for selection of RNAs that can completely envelop a ligand (something that can't be done if the ligand has a linker attached). The process only requires manipulation of DNA and proteins, obviating the need to work with chemically less-stable RNA directly. Finally, the process is amenable to automation, opening the door to fast riboswitch selection.

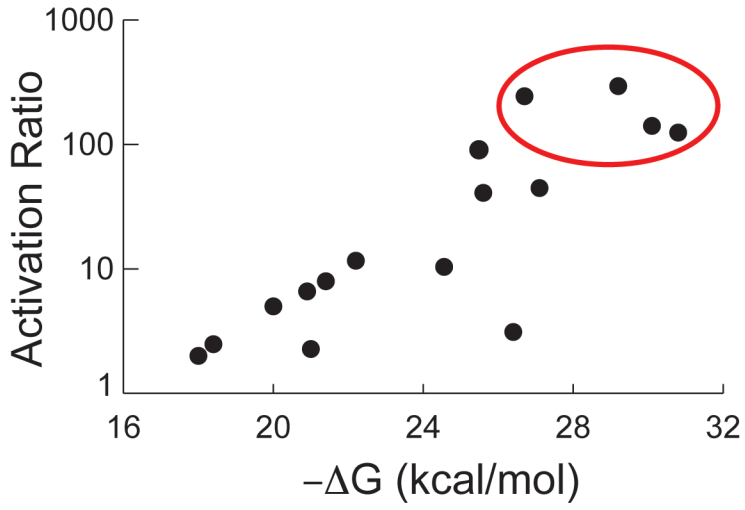

Figure 4. Plot of the observed activation ratios vs. the *mfold*-predicted ΔG of the OFF states for a set of theophylline-dependent riboswitches. Each data point represents a single riboswitch sequence. The switches corresponding to the points in the red oval were discovered using CIS-Select from a library of 65,536 possible members (Mishler, Yermakov, and Gallivan, unpublished).

Although CIS-Select has produced the best performing riboswitches that we have observed, there are still challenges associated with the procedure (which is why we have not published the method yet). Most any selection experiment will produce 'cheaters'—sequences that should not get amplified, but do. That is also the case here, however, we have not been able to discern a pattern for *why* the cheating sequences are selected. Some possibilities include insufficient washing of the solid support or dissociation of the RepA/DNA complex, followed by association with a non-cognate sequence, which would result in scrambling. We are currently investigating parameters such as time and temperature to minimize the possibility of scrambling. Another alternative would be to evolve the RepA protein to bind to DNA more tightly.

Summary

In the early granting period, we struggled to achieve the original goals of the proposal (developing riboswitches that recognize organophosphate nerve agents). By returning to first principles mechanistic studies, we were able to determine that many of our riboswitches act not as equilibrating conformers, but rather as kinetic traps. This observation dramatically changed our approach to riboswitch design—placing an increased emphasis on ligand association kinetics, and a decreased emphasis on dissociation kinetics. In addition, we developed a new method for direct riboswitch selection known as CIS-select. Although this method is still being optimized, it has many attractive advantages, including increased library sizes, fewer requirements for synthetic chemistry, and less challenging molecular biology experiments.